REMARKS

Reconsideration of the application in view of the above amendments and following remarks is respectfully requested.

Claims 7-9 and 11-12 were pending in the subject application. As set forth above, claim 12 has been cancelled without prejudice in order to expedite allowance of the remaining pending claims. No new matter has been added. Therefore, claims 7-9 and 11 are now pending in the subject application.

In the Office Action dated January 27, 2004, claim 12 was rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking adequate written description in the application.

Applicants respectfully disagree. Nevertheless, as set forth above, claim 12 has been cancelled without prejudice in order to expedite allowance of pending claims 7-9 and 11. Therefore, this rejection has been rendered moot.

In the Office Action, claims 7-9, 11 and 12 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement. Applicants respectfully disagree.

In the Office Action, it is asserted that: the claims are clearly intended to encompass methods of gene therapy and the specification is not enabling for gene therapy as a method of eliciting or enhancing an immune response against HER-2/neu; and it could not be assured that even a transient expression of the polypeptides would be achieved to the extent which would be necessary to generate an efficacious immune response. Applicants respectfully disagree.

Applicants respectfully disagree that vaccination with a nucleic acid (either directly or via a viral vector which directs the expression of the polypeptide encoded by the nucleic acid) is "gene therapy". Applicants' position is explained immediately below. Nevertheless, as described further below, even if nucleic acid vaccination is assumed to fall within "gene therapy", the invention of the presently pending claims is enabled within the meaning of Section 112, first paragraph, by the subject specification.

In the Office Action, a definition is supplied from an on-line source. Applicants note that that definition is by no means universally used. The following definitions of "gene therapy" are examples of definitions that do not encompass Applicants' claimed invention:

1. inserting the normal gene into a person, to replace a non-working or missing gene.
   www.chkd.com/Genetics/glossary.asp

2. An experimental procedure aimed at replacing, manipulating, or supplementing nonfunctional or misfunctioning genes with healthy genes.
   doegenomestolife.org/glossary/glossary_g.html

3. A therapeutic strategy aimed at introducing genes into an organism for the purpose of correcting genetic defects.
   www.nccr-oncology.ch/en/glossary/glossary.htm

4. treating disease by replacing, manipulating, or supplementing nonfunctional genes.
   www.accessexcellence.org/AE/AEPC/NIH/gene27.html

5. The treatment of genetic disease by either repairing the defective gene or replacing it with a functioning gene.
   www.fsma.org/glossary.shtml

6. An experimental procedure aimed at replacing, manipulating, or supplementing nonfunctional or misfunctioning genes with healthy genes. Source : Human Genome Project Information
   www.genomecanada.ca/prairie/glossary.asp

7. treatments that focus on replacing damaged or missing genes with healthy copies
   www.cancercare.org/managing/trials/glossary.asp

Therefore, contrary to the Office Action, it is not certain that the "claims are clearly intended to encompass methods of gene therapy".

Nevertheless, even if it is assumed that the pending claims encompass gene therapy, the claims are enabled within the meaning of Section 112, first paragraph, by the subject specification. All the pending claims are directed to methods "for eliciting or enhancing an immune response to HER-2/*neu* protein". This is all that Applicants' specification need enable. Even if the Examiner believes that the claims somehow encompass more than their recited use, it is respectfully pointed out that it is well established law that only one use has to be enabled, not all potential uses. Applicants' claimed methods do provide for eliciting or enhancing an immune response to HER-2/*neu* protein. Like most nucleic acid vaccines, the expression is transient in nature but sufficient to generate an efficacious immune response. That the claimed methods are enabled by the subject specification is confirmed by the Declaration of Cheever submitted herewith, including the journal article appended to the Declaration. (It is noted that a signed original of the Declaration will be forthcoming.) As described in the Declaration, the results shown in the article necessarily mean and confirm that sufficient polypeptide is expressed by the administered plasmid DNA encoding ICD polypeptide (intracellular domain of HER-2/neu protein) to generate an efficacious immune response. The potential problems stated in the Office Action with in vivo delivery and expression with respect to administration of nucleic acids or viral vectors were not observed with Applicants' claimed invention, as evidenced by the experiments in the article described in the Declaration. The experiments in the article described in the Declaration were performed using the teachings of the subject specification, in combination with that which was well known in the art at the time of filing with respect to general methodologies (e.g., preparation of plasmid DNA). The results from the article described in the Declaration are confirmatory that the subject specification was enabling for the claimed methods as of the time of filing. It is clear that enough expression of the claimed polynucleotides is attained and the expressed polypeptides are presented to the immune system to induce an efficacious immune response against the HER-2/*neu* protein. Accordingly, contrary to the general assertions of the Office Action, the claimed methods are enabled within the meaning of Section 112, first paragraph.

Therefore, it is believed that the rejection of claims 7-9 and 11 under 35 U.S.C. § 112, first paragraph, for lack of enablement has been overcome. Withdrawal of this rejection is respectfully requested.

Although it is believed that the enablement rejection under Section 112, first paragraph, has been overcome, Applicants wish to clarify for the record the characterization of the cited references in the Office Action. For example, Verma et al. is cited in part for difficulties in obtaining sustained expression. There are two types of expression: sustained and transient. While sustained expression is normally important where a gene is defective or missing and is being replaced, the expression for nucleic acid vaccines is always transitory and not sustained. This is because only transitory expression is necessary to start the immune system and once started the immune system reacts against the expressed polypeptide and will not permit further expression. Another example is the citation of Orkin et al., and page 8, paragraph 5, in particular. Applicants respectfully note that the last sentence of that paragraph 5 states: "However, the use of naked DNA for *in vivo* vaccination appears feasible and highly promising." Nevertheless, regardless of the characterization of the cited references in the Office Action, Applicants' claimed methods do not suffer from the problems suggested in the Office Action.

Lastly, Applicants' prior Reply and Amendment noted that certain aspects of the examination have not been consistent over the course of the prosecution of the claimed subject matter. In response, the Examiner at page 5 of the present Office Action pointed out that Office actions are done to be in consensus within the 1642 art unit as well as with related art units. While acknowledging the need for consistency at the Office, Applicants respectfully submit that it would be unfortunate if nucleic acid vaccination cases are lumped together with classic gene therapy cases where sustained expression is required and difficulties with long term replacement of genes have arisen. Any reluctance on the part of the Office to allow classic gene therapy cases should not simply be universally extended to nucleic acid vaccination cases.

Therefore, in light of the amendments and remarks set forth above, Applicants believe all the Examiner's rejections have been overcome. Reconsideration of the application and allowance of all the now pending claims (7-9 and 11) are respectfully requested. If there is any further matter requiring attention prior to allowance of the subject application, the Examiner is respectfully requested to contact the undersigned attorney (at 206-622-4900) to resolve the matter.

The Director is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

Respectfully submitted,

SEED Intellectual Property Law Group PLLC

Richard G. Sharkey

Richard G. Sharkey, Ph.D.
Registration No. 32,629

Customer Number 00500

Enclosures:

Declaration of Martin A. Cheever under 37 C.F.R. § 1.132
Foy et al., Vaccine 19:2598-2606, 2001
Notice of Appeal

MillicentS/487941

EXPRESS MAIL NO. EV449558524US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Martin A. Cheever and Mary L. Disis
Application No.	:	09/167,516
Confirmation No.	:	1422
Filed	:	October 06, 1998
For	:	COMPOUNDS FOR ELICITING OR ENHANCING IMMUNE REACTIVITY TO HER-2/*neu* PROTEIN FOR PREVENTION OR TREATMENT OF MALIGNANCIES IN WHICH THE HER-2/*neu* ONCOGENE IS ASSOCIATED

Examiner	:	Karen A. Canella, Ph.D.
Art Unit	:	1642
Docket No.	:	920010.448C8

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner:

I, Martin A. Cheever, hereby declare as follows:

1. Mary L. Disis and I are coinventors of the subject matter described in the above-identified patent application (hereinafter referred to as "subject application").

2. I hold an M.D. and was employed at the time the subject application was filed by the University of Washington where I held the position of Professor of Medicine, Division of Medical Oncology. I am presently employed by Corixa Corporation of Seattle, Washington.

3. I have been involved in immunology for over 25 years and I have authored or coauthored about 100 publications in this field. My research specialty is immunology related to cancer.

4. I am a co-author of the article appended hereto: Foy et al., Vaccine 19:2598-2606, 2001 (hereinafter referred to as "the article").

5. Figure 1 at page 2600 of the article shows that vaccination of mice with a plasmid expressing ICD polypeptide (intracellular domain of HER-2/neu protein) is effective to prevent growth of HER-2/neu tumor.

6. The results shown in Figure 1 of the article necessarily mean that sufficient polypeptide is expressed by the plasmid DNA encoding ICD polypeptide to generate an efficacious immune response.

7. The sentence beginning at the end of page 2603 and completed at the top of page 2605 of the article states: "Taken together, these data provide strong evidence to suggest that protective immunity to Her-2/neu can be elicited by vaccination, demonstrating its utility as a cancer vaccine target."

8. Thus, the results presented in the article confirm the teachings of the subject application that administration to a warm-blooded animal of DNA or plasmid expressing ICD polypeptide of HER-2/neu protein achieves transient expression to the extent necessary to generate an efficacious immune response.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

Dated this _______day of July, 2004.

____

Martin A. Cheever

MillicentS/502173

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ELSEVIER

Vaccine 19 (2001) 2598–2606

Vaccine

www.elsevier.com/locate/vaccine

Vaccination with Her-2/neu DNA or protein subunits protects against growth of a Her-2/neu-expressing murine tumor

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Corixa Corporation, 1124 Columbia St. Suite 200, Seattle, WA 98104, USA

Abstract

The present study utilizes an in vivo murine tumor expressing human Her-2/neu to evaluate potential Her-2/neu vaccines consisting of either full length or various subunits of Her-2/neu delivered in either protein or plasmid DNA form. Our results demonstrate that protective immunity against Her-2/neu-expressing tumor challenge can be achieved by vaccination with plasmid DNA encoding either full length or subunits of Her-2/neu. Partial protective immunity was also observed following vaccination with the intracellular domain (ICD), but not extracellular domain (ECD), protein subunit of Her-2/neu. The mechanism of protection elicited by plasmid DNA vaccination appeared to be exclusively CD4 dependent, whereas the protection observed with ICD protein vaccination required both CD4 and CD8 T cells.

1. Introduction

Her-2/neu is a proto-oncogene encoding a 185 kD protein with homology to the epidermal growth factor receptor (EGFR) family [1,2]. It is over-expressed in several human cancers including breast, ovarian, and gastric carcinomas [3,4]. Studies of patients with Her-2/neu expressing tumors have demonstrated the existence of *anti*-Her-2/neu antibody and T cell responses [5–8], yet in most cases, the immune responses in these patients is insufficient to provide protection against tumor growth. Other groups have demonstrated that Her-2/neu-specific cytotoxic T cells (CTL) or T helper cells (Th) can be generated through in vitro stimulation of T cells with autologous Her-2/neu-expressing tumor cells or Her2-neu derived peptides, indicating that in some patients the potential exists for boosting *anti*-Her-2/neu immunity [5,7,9–11]. Taken together, these studies provide support for Her-2/neu as a candidate for a tumor antigen vaccine.

The only current approved Her-2/neu immunotherapy is passive transfer of a Her-2/neu monoclonal antibody (reviewed in [12]). Although this has been shown to be effective at inhibiting tumor growth in a limited population of Her-2/neu patients, it is possible that elicitation of an active and more comprehensive immune response that includes both antibody and T cell responses may provide more effective anti-tumor immunity. The present study utilizes an in vivo murine tumor expressing human Her-2/neu to evaluate several potential Her-2/neu vaccines. In this study we have evaluated vaccines consisting of either full length or various subunits of Her-2/neu delivered in either protein or plasmid DNA form. Our results demonstrate that protective immunity against Her-2/neu-expressing tumor challenge can be achieved by vaccination with plasmid DNA encoding either full length or subunits of Her-2/neu. Partial protective immunity was also observed following vaccination with the intracellular domain (ICD), but not extracellular domain (ECD), protein subunit of Her-2/neu. Interestingly, the mechanism of protection elicited by plasmid DNA vaccination appeared to be exclusively CD4 dependent, whereas the protection observed with ICD protein vaccination required both CD4 and CD8 T cells.

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0264-410X/01/$ - see front matter
PII: S0264-410X(00)00493-X

2. Materials and methods

2.1. Animals

Eight to 12 week old female C57Bl/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in our animal facility at Corixa Corporation.

2.2. Antibodies and reagents

Rat *anti*-murine CD4 (GK1.5) and rat *anti*-murine CD8 (2.43) hybridoma cell lines were obtained from ATCC. Antibody was purified from ascites fluid. Ab-5, an *anti*-human Her-2/neu ECD-specific antibody, was purchased from Oncogene Research Products (Cambridge, MA). Montanide 720 was purchased from Seppic Inc. (Fairfield, NJ).

2.3. Tumor cell lines

EL4, a murine thymoma originally derived from C57BL mice, was obtained from ATCC. EL4 cells were transfected with full length human Her-2/neu using a standard electroporation protocol. EL4 cells stabely expressing Her-2/neu were obtained following in vitro drug selection with neomycin. Her-2/neu expression was confirmed by flow cytometric analysis.

2.4. Her2/neu vaccines

Her-2/neu plasmid DNA vaccine (pVR1012-Her-2/neu) consisted of the full length human Her-2/neu cDNA inserted into VR1012 (Vical, San Diego, CA). The ECD plasmid DNA vaccine (pVR1012-ECD) consisted of DNA encoding amino acids 1–695 of Her-2/neu and the ICD plasmid DNA vaccine (pVR1012-ICD) consisted of DNA encoding amino acids 692–1256 in VR1012. Large quantities of endotoxin free plasmid DNA were prepared using Qiagen Inc. (Valencia, CA) kit reagents and standard techniques. Plasmid DNA vaccines were delivered intramuscularly (100 ug) on day 0 and 21. ICD (amino acids 676–1256) and ECD (amino acids 22–653) recombinant subunit proteins were produced at Corixa Corporation. Briefly, ECD protein was produced by stable transfection of L cells and purified using a combination of DEAE, reverse phase HPLC, and Mono S column chromatography. ICD protein was produced in *E. coli* and purified from solubilized inclusion bodies via High Q anion exchange, followed by nickel resin affinity chromatography. Recombinant protein vaccines were mixed with Montanide 720 at a 7:2 (Montanide 720:protein) ratio and delivered subcutaneously.

2.5. In vivo tumor model

To ensure a consistent source of EL4-Her-2/neu cells for tumor protection experiments, cells were expanded by in vivo passage (i.p.) and frozen in aliquots for use in individual experiments. Tumors were established using 200 000 EL4-Her-2/neu cells injected subcutaneously on the flank. Palpable tumors typically developed within 8–10 days of injection. Tumor size is expressed in mm^2 as determined by measuring the area (length × width) of the tumors with a microcaliper device.

2.6. In vivo depletion of effector T cells

Mice were immunized with plasmid DNA or protein on days 0 and 21 to generate effector T cells. CD4 and CD8 cells were depleted by i.p. administration of 100 ug per day of purified *anti*-CD4 or *anti*-CD8 antibody on day 35, 38, and 42 following initiation of the experiment. Flow cytometric analysis of depleted splenocytes indicated greater than 98% depletion of the target populations.

2.7. Adoptive transfer of immune sera

Immune sera were obtained through bleeds of Her-2/neu plasmid DNA- or ICD protein-immunized mice. Sera from 12 individual mice from each group were pooled for transfer (i.v.) into six naïve recipient mice. Anti-Her-2/neu antibody titers of immune sera were assessed by ELISA prior to sera transfer.

2.8. In vitro cytokine analysis

Mice (four per group) were immunized with 100 ug pVR1012 or pVR1012-Her-2/neu (i.m) or 50 ug of ICD protein in Montanide (s.q.), or Montanide alone on day 0 and 21. Two weeks following the second immunization, 250 000 spleen cells were harvested and stimulated in vitro with media alone, ICD or ECD protein (10 μg/ml). IFNγ secretion was assayed by ELISA from supernatants taken 48 h following in vitro stimulation. Values represent the mean of tripicate wells for four individual mice.

3. Results

3.1. Her-2/neu protein subunit and plasmid DNA vaccines mediate tumor protection

Her 2/neu vaccines consisting of either full length or truncated forms of Her-2/neu were evaluated for the ability to elicit a protective immune response against challenge with a syngeneic Her-2/neu expressing tumor

cell line. C57Bl/6 mice were immunized with plasmid DNA encoding full length human Her-2/neu, ICD, or the ECD portions of Her-2/neu. Following two DNA immunizations, mice were challenged subcutaneously with EL4 cells transfected with full length human Her-2/neu (EL4-Her-2/neu) and tumor growth was monitored. In naïve mice, EL4-Her-2/neu cells form large solid tumors within 14–20 days of subcutaneous administration. Vaccination with Her-2/neu plasmid DNA, either full length, ICD or ECD subunits, substantially inhibits the growth of the tumor cells (Fig. 1). The majority of mice are completely protected from developing tumor, whereas as small portion of animals demonstrate a delay in tumor development for up to 3 weeks following tumor challenge. It is interesting to note that similar levels of tumor protection are achieved with both the truncated and the full length Her-2/neu constructs.

To determine whether protein subunit vaccines were also effective at eliciting tumor protection, mice were immunized with ICD or ECD protein plus adjuvant, challenged with EL4-Her-2/neu, and monitored for tumor growth. The results, shown in Fig. 2, demonstrate that vaccination with ICD protein elicits a partially protective immune response in which both the frequency of mice developing tumor and the mean tumor size of mice bearing tumors are decreased. In this representative experiment, ICD vaccination results in complete protection of one animal, and a decrease in mean tumor size of the mice developing tumors (162 mm^2 on d23). This is compared to tumor growth in 4/4 mice (mean tumor size of 527 mm^2) in the naïve group and 4/4 mice (mean tumor size of 462 mm^2) in the ECD vaccinated group. Although the protection observed with ICD protein is only partial, we consistently observed a decrease in tumor size or complete protection in the majority of mice vaccinated with ICD protein. In comparison to Her-2/neu DNA vaccination however, it is clear that with the formulation and regimen employed protein vaccination is not as efficacious as plasmid DNA.

In order to determine whether the protection observed in this model was Her-2/neu specific, mice were vaccinated with full length Her-2/neu, or vector control plasmid DNA, and subsequently challenged with either parental EL4 or EL4-Her-2/neu cells. Growth of the tumors was monitored over the next 10–25 days. The results, depicted in Fig. 3, demonstrate that prevention of tumor growth only occurs in mice immunized with Her-2/neu plasmid DNA, suggesting that immunity to Her-2/neu is elicited and required for protection. Further evidence that tumor protection is Her-2/neu specific is provided by the observation that vaccination with Her-2/neu plasmid DNA does not prevent growth of the parental EL4 cells. Similar results were observed when ICD protein was used as the vaccine (data not

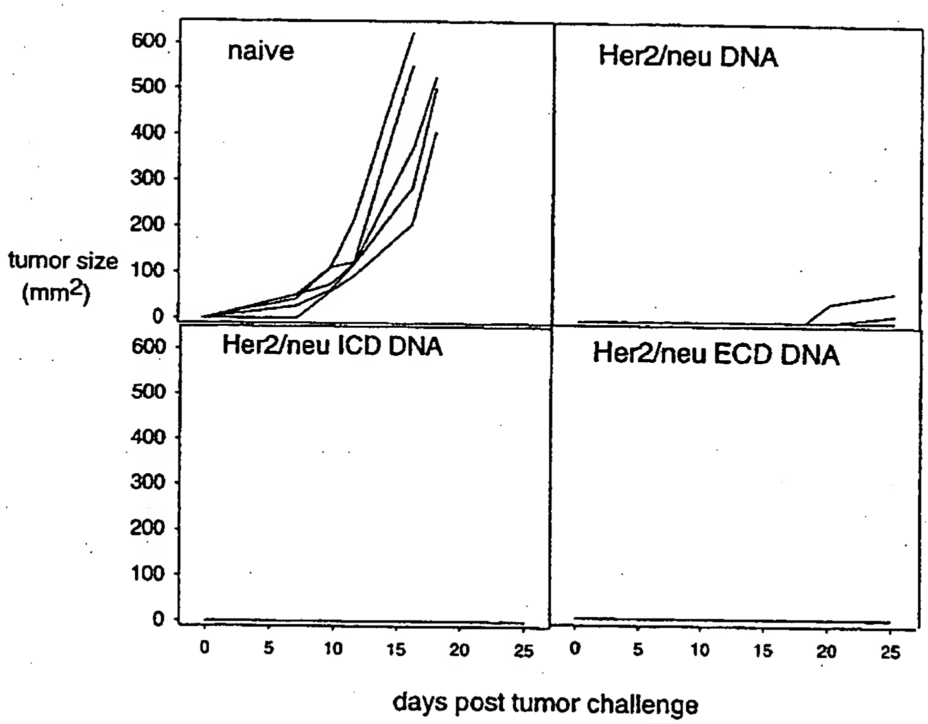

Fig. 1. Growth of EL4-Her-2/neu is inhibited by vaccination with plasmid DNA encoding Her-2/neu. Mice (five per group) were immunized (i.m.) with pVR1012-Her-2/neu, pVR1012-ECD or pVR1012-ICD (100 ug) on day 0 and 21. Mice were challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 35. Tumor size was monitored for 25 days following tumor challenge.

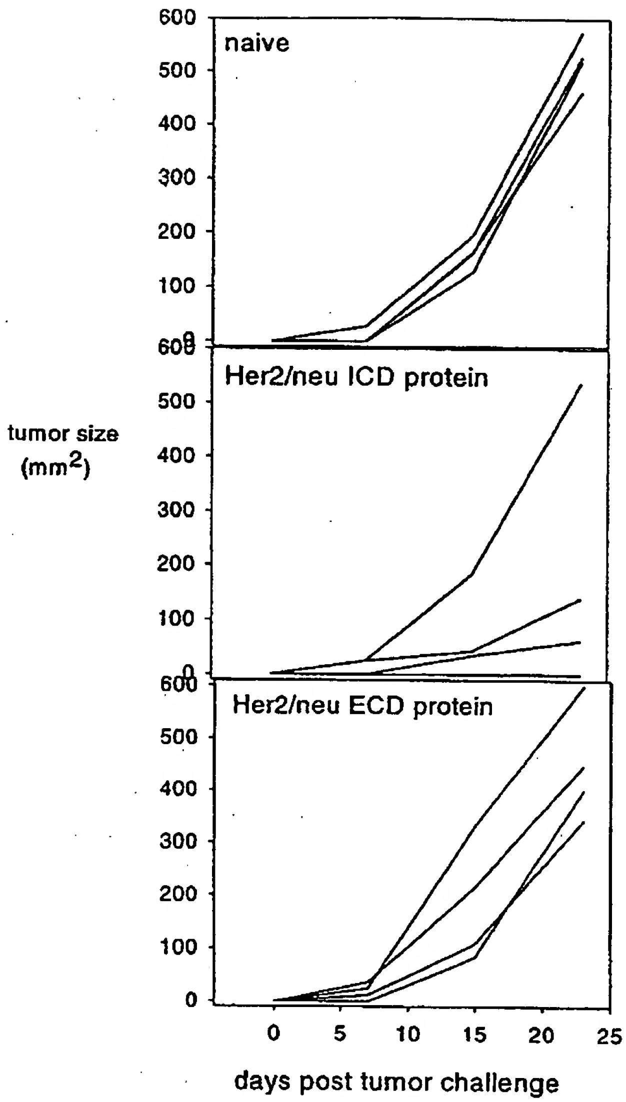

Fig. 2. Growth of EL4-Her-2/neu is partially inhibited by vaccination with the Her-2/neu ICD, but not ECD protein subunit. Mice (four per group) were immunized (s.q.) with Her-2/neu ICD or Her-2/neu ECD protein (50 ug) in Montanide 720 on day 0 and 21. Mice were challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 35. Tumor size was monitored for 25 days following tumor challenge.

shown). Taken together, these results indicate that tumor protection mediated by Her-2/neu vaccines is Her-2/neu specific.

3.2. Mechanism of tumor protection mediated by Her-2/neu protein subunits or plasmid DNA vaccines

In order to determine the nature of the immune response responsible for mediating tumor protection with Her-2/neu plasmid DNA or protein vaccination we next performed a series of in vivo depletion and adoptive transfer experiments. The first experiments were designed to evaluate the respective roles of CD4 and CD8 effector T cells. Mice were immunized twice with full length Her-2/neu or control plasmid DNA. Two weeks following the second immunization, mice were treated in vivo with *anti*-CD4 or *anti*-CD8 antibodies to deplete effector T cells. Greater than 98% CD4 or CD8 splenic T cell depletion was achieved by three administrations of antibody over the course of 7 days. Three days later, mice were challenged with EL4-Her-2/neu and monitored for tumor growth. Fig. 4 depicts the results of one representative experiment in which CD4 and CD8 effector T cells were depleted following Her-2/neu DNA vaccination. Consistent with the previous experiment shown in Fig. 1, complete tumor protection is observed in mice which were vaccinated with Her-2/neu plasmid DNA (untreated group). In contrast, in vivo depletion of CD4, but not CD8, effector T cells completely abrogates tumor protection mediated by Her-2/neu DNA vaccination. Similar results were obtained in adoptive transfer experiments where it was observed that adoptive transfer of CD8-, but not CD4-depleted effector T cells conferred protection against tumor challenge (data not shown). Collectively, these results suggest that in this tumor model, protection mediated by plasmid DNA vaccination is dependent upon the presence of CD4, but not CD8 effector T cells.

Similar experiments were carried out following vaccination with ICD protein to determine the roles of CD4 and CD8 T cells in the immune response elicited by this vaccine. Again, mice were immunized and boosted with ICD protein in adjuvant, depleted of CD4 and CD8 effector T cells by in vivo antibody treatment, and subsequently challenged with EL4-Her-2/neu. The results, shown in Fig. 5, indicate that depletion of either CD4 or CD8 T cells abrogates the partial protection obtained with ICD vaccination, suggesting that both CD4 and CD8 effector T cells play a role in ICD protein-mediated tumor protection. Results of adoptive transfer experiments also indicate that both CD4 and CD8 effector cells are important in the immune response elicited by ICD protein vaccination (data not shown).

Because it is known that *anti*-Her-2/neu antibodies can exhibit *anti*-proliferative effects on tumor cells, we investigated whether antibodies elicited by either plasmid DNA or protein vaccination contributed to the observed protection. In order to address this question, mice were immunized and boosted with full length Her-2/neu DNA or ICD protein. Sera from Her-2/neu immune mice or control sera from non-immune mice were collected and then transferred into naïve mice which were then challenged with EL4-Her-2/neu. The results from Her-2/neu DNA immune sera, shown in Fig. 6, indicate that transfer of antibody does not confer protection. These results are somewhat predictable given that the levels of *anti*-Her-2/neu antibod-

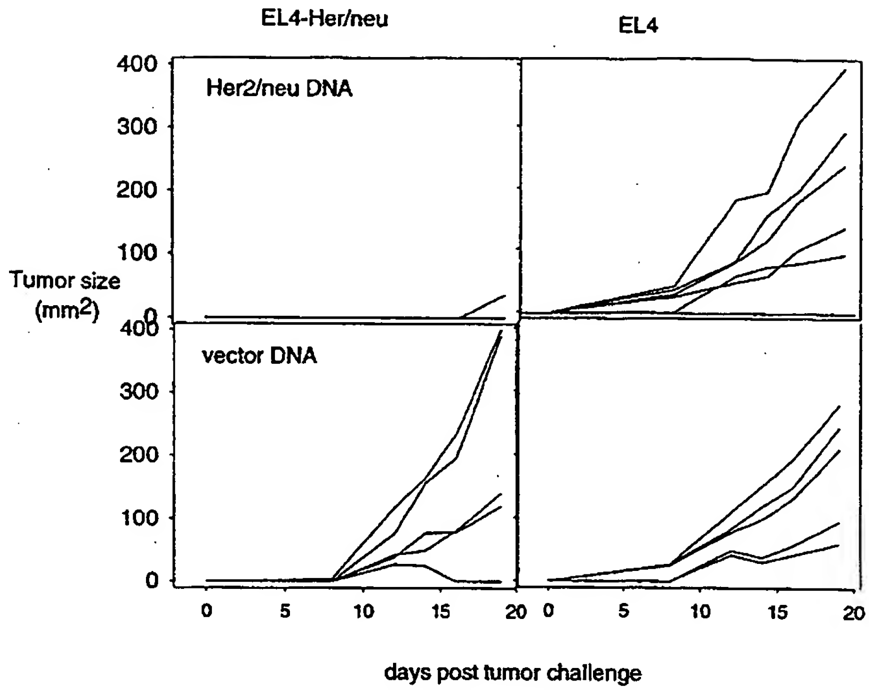

Fig. 3. Tumor protection is Her-2/neu specific. Mice (five per group) were immunized i.m. with pVR1012 or pVR1012-Her-2/neu (100 ug) on day 0 and 21. Mice were challenged with 200 000 parental EL4 cells or EL4-Her-2/neu cells subcutaneously on day 35. Tumor size was monitored for 25 days following tumor challenge.

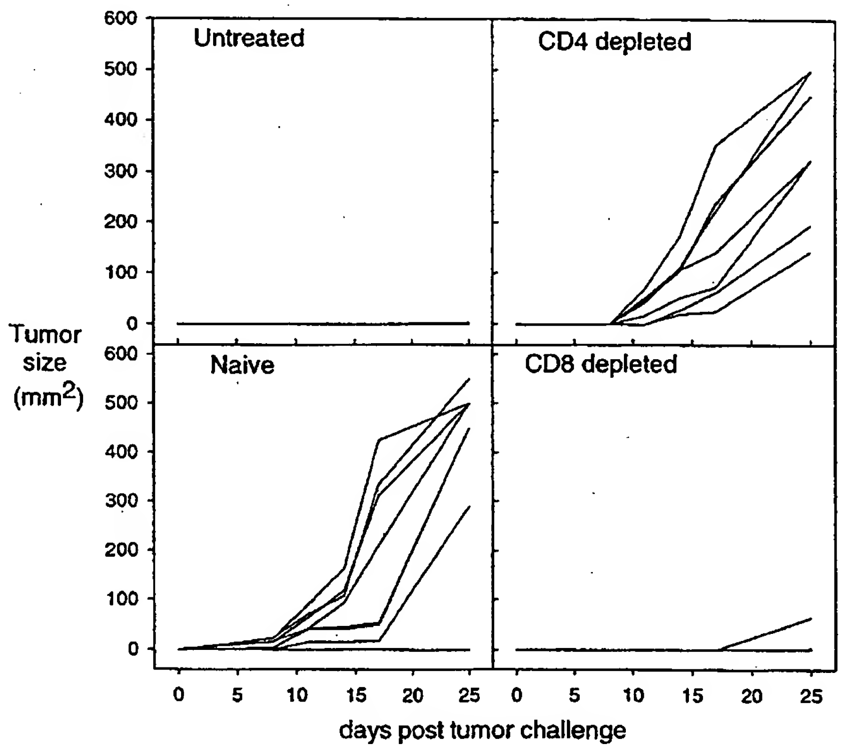

Fig. 4. Tumor protection mediated by Her-2/neu plasmid DNA vaccination requires CD4 T cells. Mice (eight per group) were immunized (i.m.) with pVR1012-Her-2/neu on day 0 and 21. On day 35, 38, and 42 mice were injected i.p. with 100 ug *anti*-CD4 or *anti*-CD8 antibodies to deplete effector T cells. Mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 45. Tumor size was monitored for 25 days following tumor challenge.

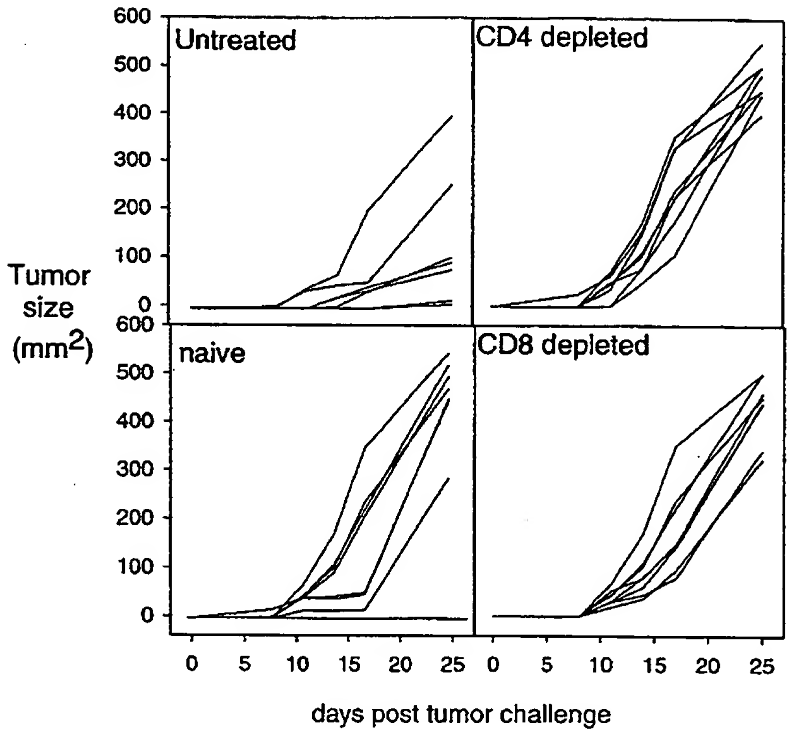

Fig. 5. Tumor protection mediated by Her-2/neu ICD protein subunit vaccination involves both CD4 T and CD8 T cells. Mice (eight per group) were immunized (s.q.) with Her-2/neu ICD protein (50 ug) in Montanide 720 on day 0 and 21. On day 35, 38, and 42 mice were injected i.p. with 100 ug *anti*-CD4 or *anti*-CD8 antibodies to deplete effector T cells. Mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously on day 45. Tumor size was monitored for 25 days following tumor challenge.

ies obtained with plasmid DNA vaccination are quite low (data not shown). Similarly, transfer of *anti*-ICD containing sera was not protective (Fig. 7), despite the presence of substantial titers (10 000–100 000) of *anti*-ICD antibody present in this sera (data not shown). Taken together, these results suggest that antibody does not mediate the protection observed in this model using EL4-Her-2/neu tumor cells.

Results of these in vivo depletion and adoptive transfer experiments indicate that CD4+ T cells play a major role in the elicitation of a protective anti-tumor immune response in this model. In order to more fully elucidate the mechanism by which CD4+ T cells mediate protection, we examined the cytokine secretion profile of T cells following vaccination with either Her-2/neu DNA or ICD protein. The results, summarized in Table 1, demonstrate that upon in vitro restimulation with recombinant ICD or ECD protein, spleen cells from Her-2/neu plasmid DNA vaccinated mice secrete substantial levels of IFNγ compared to unstimulated cells. Spleen cells from ICD protein vaccinated mice also produced IFNγ in response to in vitro stimulation with ICD, but as expected, not ECD protein. The levels of IL4 and IL-5 in these same cultures were below detection, consistent with a Th1-type immune response. Taken together, these results suggest that IFNγ may play a role in the protection mediated by our Her-2/neu vaccines in this model.

4. Discussion

The present study demonstrates the utility of a Her-2/neu vaccine for the inhibition of tumor cell growth in vivo. We have shown that immunization with plasmid DNA encoding either full length or subunits of Her-2/neu effectively prevents growth of EL4-Her-2/neu in vivo in an antigen specific manner. Furthermore, our results indicate that protection mediated by Her-2/neu DNA is predominately CD4 and not CD8 or antibody dependent. Similar experiments carried out to evaluate Her-2/neu protein subunit vaccines indicate that ICD, but not ECD protein vaccination elicits partial tumor protection. In contrast to plasmid DNA-induced immunity, tumor protection observed with ICD protein vaccination involves both CD4 and CD8 effector T cells. Finally, analysis of the antigen-specific IFNγ production following protein and DNA immunization suggests that IFNγ is likely to play a role in elimination of Her-2/neu expressing tumor cells in vivo. Taken together, these data provide strong evidence to suggest

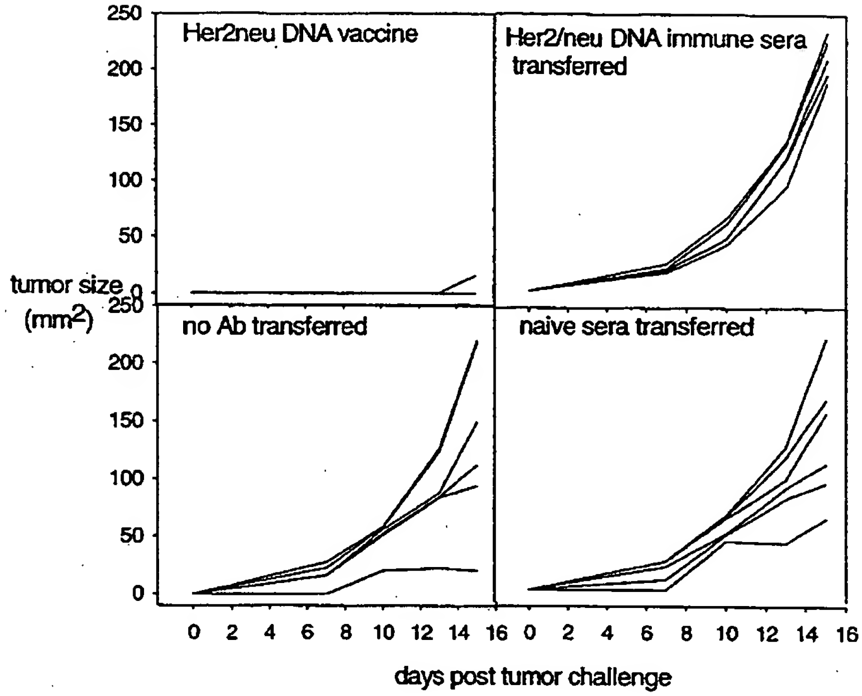

Fig. 6. Tumor protection mediated by Her-2/neu plasmid DNA vaccination is not antibody dependent. Mice (12 per group) were immunized (i.m.) with pVR1012-Her-2/neu on day 0 and 21. On day 35 immune sera were collected from the mice, pooled and transferred into six naïve recipient mice. Recipient mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously. Tumor size was monitored for 25 days following tumor challenge.

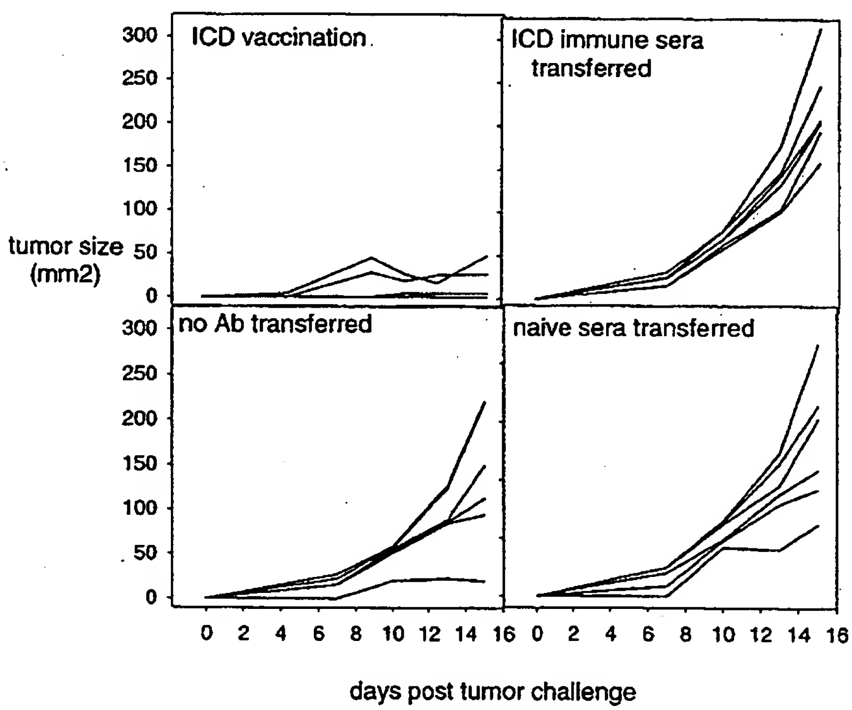

Fig. 7. Tumor protection mediated by Her-2/neu ICD protein subunit vaccination is not antibody dependent. Mice (12 per group) were immunized (s.q.) with Her-2/neu ICD protein (50 ug) in Montanide 720 on day 0 and 21. On day 35 immune sera were collected from the mice, pooled and transferred into six naïve recipient mice. Recipient mice were then challenged with 200 000 EL4-Her-2/neu cells subcutaneously. Tumor size was monitored for 25 days following tumor challenge.

Table 1
IFNγ production following Her-2/neu DNA or protein vaccination

Vaccine[a]	IFNγ (ng/ml)		
	Medium[b]	ICD	ECD
pVR1012-Her-2/neu	0.32[c]	4.17	1.27
pVR1012	0.61	0.36	0.42
ICD protein	0.31	2.16	.01
Adjuvant alone	1.30	1.23	1.35

[a] Mice (four per group) were immunized with 100 ug pVR1012 or VR1012-Her-2/neu (i.m) or 50 ug of ICD protein in Montanide (s.q.), or Montanide alone on day 0 and 21.

[b] Two weeks following the second immunization, spleen cells were harvested and stimulated in vitro with media alone, ICD or ECD protein (10 ug/ml).

[c] IFNγ secretion was assayed by ELISA 48 h following in vitro stimulation. Values represent the mean of tripicate wells for four individual mice.

that protective immunity to Her-2/neu can be elicited by vaccination, demonstrating its utility as a cancer vaccine target.

In the present study, we chose to evaluate vaccines consisting of truncated forms or subunits of Her-2/neu because of the potential safety concerns regarding the use of a functionally active form of an oncogene in a vaccine. Her-2/neu, a known proto-oncogene has the potential to transform cells expressing it and therefore it may be difficult to gain approval for its use in humans. The results of plasmid DNA vaccine experiments demonstrate that tumor protection is effectively achieved using all three forms of Her-2/neu, supporting the contention that safety issues can be circumvented through the use of non-functional, truncated forms of the Her-2/neu antigen without compromising the quality of the protective response. These results are consistent with those obtained by other groups demonstrating protective immunity following plasmid DNA immunization with neu in FVB/N rat neu transgenic mice [13–15]. Our results also support those of Wei et al. who demonstrated protection using a mutated form of Her-2/neu in which the kinase activity had been eliminated [16]. More importantly, our data extend their observation to show that protective immunity can be achieved with smaller, truncated versions of Her-2/neu.

One primary aim of the current study was to determine the mechanism by which our Her-2/neu vaccines confer anti-tumor immunity. Numerous studies have established that both CD4+ and CD8+ T cells are required to achieve systemic anti-tumor immunity, whereas antibodies have been thought to play a lesser role. It has been proposed that CD8+ T cells are the predominant effector cells involved in anti-tumor immune responses (reviewed in [17]) and that CD4+ T cells are important for providing help to CD8+ T cells during priming [18–20]. There is a growing body of evidence, however, to suggest that CD4+ T cells can also play a direct role in mediating tumor immunity [21–24]. In the model presented in this study, T cells are clearly the major effector mechanism mediating tumor protection. This appears to be the case regardless of the composition of the Her-2/neu vaccine (i.e. plasmid DNA or protein). It is interesting to note, however, that the effector T cell population(s) responsible for protection mediated by DNA and protein are distinct. CD4+ T cells are the major effectors for plasmid DNA mediated protection, whereas both CD4+ and CD8+ T cells seem to be important in tumor protection following protein vaccination.

The observation that CD4+ T cells are the primary effector cells generated as a result of DNA vaccination is not without precedence. Although it is well documented that intramuscular plasmid DNA vaccines are very efficient at eliciting CTL responses (reviewed in [25]), evidence suggests that elicitation of CD4+ T-helper type 1 (Th1) T cell responses is also a property of DNA vaccines [26]. Moreover, there is increasing evidence to support the role of CD4+ T cells of both the Th1 and Th2 types in mediating anti-tumor immunity by indirect mechanisms. Mumberg et al. demonstrated that CD4+ T cells can eliminate MHC class II-negative tumor cells in vivo by an indirect IFNγ-dependent mechanism which likely stimulates other host cells to become tumorcidal [27]. Similarly, Hung et al. confirmed that cytokine production by CD4+ T cells plays an essential role in the recruitment of other anti-tumor effector cells such as macrophages and eosinophils in vivo [24]. Our observation that IFNγ secretion is readily detectable upon in vitro restimulation of immune spleen cells from Her-2/neu DNA-immunized mice suggests that CD4+T cell secretion of IFNγ may likely be a component of the protective mechanism in this model. Current studies are underway to more specifically define the role of CD4+ T cells in this model.

Tumor protection resulting from protein immunization in this model appears to require both CD4+ and CD8+ T cells, indicating perhaps a more conventional role for CD4+ T cells in providing help to CD8+ T cells following protein vaccination. However, we were unable, despite exhaustive efforts, to detect Her-2/neu specific CTL following immunization in this model, suggesting that Her-2/neu specific CD8 T cells may be at a low precursor frequency, or alternatively, may be mediating protection via an effector mechanism other than direct anti-tumor cytolysis. Finally, although it is unclear why vaccination with the ICD subunit protein provides tumor protection when the ECD subunit protein does not, the observation that both proteins elicit detectable antibody responses supports the contention antibody does not mediate protection in this model.

The present study demonstrates the utility of multiple forms of Her-2/neu vaccines for the elicitation of protective Her-2/neu specific anti-tumor responses. Current studies are underway to comparatively evalaute these distinct formulations in primate models in an attempt to develop vaccines useful for treating human Her-2/neu expressing carcinomas.

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